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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/85, 9/00, 15/86, 15/11, A61K 31/70, 48/00 | A2

(11) International Publication Number: **WO 98/48027**

(43) International Publication Date: 29 October 1998 (29.10.98)

(21) International Application Number: PCT/US98/08003

(22) International Filing Date: 21 April 1998 (21.04.98)

(30) Priority Data:

60/044,492	21 April 1997 (21.04.97)	US
60/046,146	9 May 1997 (09.05.97)	US

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(81) Designated States: AL, AU, BA, BB, BG, BR, CA, CN, CU, CZ, EE, GE, GW, HU, ID, IL, IS, JP, KP, KR, LC, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, SL, TR, TT, UA, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

Without international search report and to be republished upon receipt of that report.

(54) Title: MATERIALS AND METHODS FOR TREATMENT OF RETINAL DISEASES

(57) Abstract

The subject invention provides materials and methods for efficient, specific expression of proteins in retinal photoreceptors cells. Specifically, the constructs are composed of an adeno–associated viral vector contacting a rod or cone–opsin promoter. These materials and methods can be used in therapies for retinal diseases. In one embodiment, ribozymes which degrade mutant mRNA are used to treat retinitis pigmentosa.

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DESCRIPTION

MATERIALS AND METHODS FOR TREATMENT OF RETINAL DISEASES

The subject invention was made with government support under a research project supported by NIH Grant Nos. EY07864 and EY11123 . The government has certain rights in this invention.

Cross-Reference to Related Applications

This application claims priority from provisional applications USSN 60/046,147, filed May 9, 1997; and USSN 60/044,492, filed April 21, 1997.

Background of the Invention

Retinitis pigmentosa (RP) is a collection of heritable retinal degenerations caused by defects in one of several genes for proteins of photoreceptor (PR) cells. RP is a clinically and genetically heterogeneous group of conditions characterized by progressive rod photoreceptor degeneration and eventual blindness. Of the nearly 100 causative gene defects currently known, all are either directly or indirectly involved in the rod cell specific visual response. The exact molecular pathogenesis of RP is still unexplained. Ultrastructural observations suggest that the rod PRs are severely affected in the disease. RP families have been documented with dominant, recessive, X-linked, and digenic patterns of inheritance, and more than fifteen separate loci have been implicated by linkage studies. Currently, the mutations identified to date all occur in genes exhibiting a PR-specific pattern of expression. Approximately 50,000 individuals in the United States are estimated to have RP.

Macular degeneration is a deterioration of the macula (the cone-rich center of vision) leading to gradual loss of central vision. Eventual loss of these cones leads to central vision loss and functional blindness. Macular degeneration may also have a genetic etiology or predisposition. Although a genetic etiology has not yet been established, based on pedigree studies it is very likely to exist. At least 500,000 individuals are estimated to suffer from macular degeneration currently in the United States.

There is currently no effective treatment for most forms of retinitis pigmentosa or macular degeneration. Treatment with a massive supplement (15,000 I.U. per day) of vitamin

A often retards the course of retinal degeneration in retinitis pigmentosa. Vitamin therapy does not treat the underlying cause of RP and is not a cure.

Recombinant AAV vectors have been reported to efficiently transduce central nervous system (Kaplitt, M., P. Leone, R. Samulski, X. Kigo, D. Pfaff, K. O'Mally, M. During (1993) *Nature Genetics* 8:148-154), lung (Aflone, S., C. Conrad, W. Kearns, S. Chunduru, R. Adams, T. Reynolds, W. Guggino, G. Cutting, B. Carter, T. Flotte (1996) *J. Virol.* 70:3235-3241; Flotte, T., S. Afione, C. Conrad, S.A. McGrath, R. Solow, H. Oka, P.R. Zeitlin, W.B. Guggino, C. Bi (1993) *Proc. Natl. Acad. Sci. USA* 90:10613-10617), and muscle (Xiao, X, J. Li, R. Samulski (1996) *J. Virol.* 70:8098-8108; Kessler, P., G. Podsakoff, X. Chen, S. McQulstron, P. Colosi, L. Matelis, G. Kurtzman, B. Byrne (1996) *Proc. Natl. Acad. Sci. USA* 93:14082-14087). Although a recent report (Kido, M., K. Rich, G. Lang, E. Barron, D. Kohn, M. Al-Ubaidi, J. Blanks (1996) *Curr. Eye Res.* 15:333-344) describes using an opsin promoter in a recombinant retrovirus for *ex vivo* transduction of cultured cells, dislocated retinal cells and fetal mouse retinal explants, the efficiency was very low (ca. < 0.1%).

Brief Summary of the Invention

The subject invention concerns materials and methods for achieving expression of proteins in retina cells. The expression of proteins in retina cells can be used, for example, for the treatment of retinal diseases. More specifically, the subject invention provides polynucleotide sequences, and methods for using these sequences, to achieve highly specific expression of proteins in the retina. As described herein, the expression of these proteins can be used to treat a variety of retinal diseases. In an embodiment specifically exemplified herein, the materials and methods of the subject invention can be used to treat autosomal dominant retinitis pigmentosa (ADRP).

In one embodiment, the subject invention provides techniques for obtaining targeted, high level expression of any desired gene in the photoreceptor cells of the retina. These methods involve the use of specific sets of promoter sequences that allow RNA transcription of the delivered gene exclusively in retinal rod and/or cone photoreceptor cells. Rods and cones are the principal cell types affected in retinitis pigmentosa and macular degeneration respectively, hence the ability to target expression of therapeutic genes to these cells without altering unaffected cell types in the retina provides a genetic therapy approach of high specificity and low risk.

In a specific embodiment of the subject invention, ribozymes can be highly and specifically expressed in the retina. The ribozymes cleave the mutant forms of messenger RNA

(mRNA) occurring in common forms of inherited retinal degeneration. This specificity makes these ribozymes able to destroy harmful mRNA while leaving normal mRNA intact. Ribozymes against other genetic forms of retinitis pigmentosa can be produced and used according to the subject invention. Other polynucleotides encoding therapeutically useful products can also be selectively expressed in the eye using the teachings of the subject invention.

Brief Summary of the Drawings

Figures 1a and 1b show the construction of plasmids used according to the subject invention. These figures show a schematic diagram of the plasmid DNA constructs used to make rAAV viruses mOp-*lacZ* (a) and mOp-*gfp* (b). TR, 145 bp AAV terminal repeat sequence, mOp, 472 bp murine rod opsin regulatory sequence from +86 to -388, SD/SA, 180 bp SV40 late viral protein gene 16S/19S splice donor and acceptor signal, *lacZ*; coding sequence for the bacterial *lacZ* gene; *gfp*, coding sequence for the synthetic green fluorescence gene; pA, pA1 and pA2, polyadenylation signals; Epo, a tandem repeat of the polyoma virus enhancer region (bases 5210-5274); Ptk, thymidine kinase promoter of herpesvirus (bases 92-218); neo^r, coding sequence of the neomycin resistance gene, Tn5 (bases 1555-2347) (Zolotukhin, S., M. Potter, W. Hauswirth, J. Guy, N. Muzyczka (1996) *J. Virol.* 70:4646-4654).

Figures 2a-2b show outer retinal layer with P23H ribozymes. **2a**, measurements of ONL thickness (left), RIS length (middle), and ROS length (right) in rats killed at different ages. Filled squares denote normal, non-transgenic animals. P23H-3 rats were either uninjected (open squares), injected subretinally with PBS (open diamonds), or injected with AAV vectors carrying one of five ribozymes or controls. Ribozymes were: Hp11 hairpin ribozyme (filled circles), Hh13 hammerhead ribozyme (filled triangles), Hp11i "inactive" hairpin ribozyme (open circles), Hh13i "inactive" hammerhead ribozyme (open triangle), or BOPS-*gfp* (X), all regulated by the same bovine opsin promoter. All injections were performed at P14-15. The error bars were omitted if they fell within the symbol, except for Hp11i at P75 and P90, where only one eye at each point was examined. **2b**, Measurements of ONL thickness along the vertical meridian of the eye from the optic nerve head (ONH) to the ora serrata (anterior margin of the retina) in rats at P90. Rats were either uninjected (open triangles) or injected at P14-15 with Hp11 hairpin ribozymes (filled circles) or Hh13 hammerhead ribozymes (open circles).

Detailed Disclosure of the Invention

The subject invention pertains to materials and methods for achieving highly specific expression of desired proteins in the retina. These proteins can be used as described herein to

achieve a beneficial therapeutic affect. In one aspect, the subject invention provides materials and methods which can be used to reduce or eliminate the symptoms of inherited eye disease caused by mutations in genes for retinal proteins. To this end, the subject invention provides materials and methods for achieving efficient and cell type-specific expression of exogenous genes in photoreceptor cells (PRs) of the mammalian retina. In a specific embodiment of the subject invention, recombinant Adeno-associated Virus (rAAV) vectors can be used to transfer the desired genes to retina cells.

In a specific embodiment, the subject invention provides a method for treating autosomal dominant retinitis pigmentosa (ADRP) at a molecular level. Gene therapy for ADRP according to the subject invention involves (1) an efficient and cell-type specific gene delivery/expression system, and (2) a selective means of inhibiting production of the mutant protein.

Provided herein are the results of experiments wherein rAAV vectors are used to transfer the bacterial *lacZ* gene or a synthetic green fluorescent protein gene (*gfp*) to mouse or rat retinas following injection into the subretinal space. These results demonstrate the surprising and advantageous ability to achieve highly specific expression of proteins in the mammalian retina. For example, employing a proximal murine rod opsin promoter (+86 to -385) to drive expression, reporter gene product was found exclusively in photoreceptors, not in any other retinal cell type or in the adjacent retinal pigment epithelium (RPE). GFP-expressing photoreceptors typically encompassed 10-20% of the total retinal area following a single 2 μl injection. Both rod and cone photoreceptors were transduced with nearly 100% efficiency in the region directly surrounding the injection site. Approximately 2.5 million photoreceptors were transduced as a result of the single subretinal inoculation. The use of such proximal opsin promoters therefore can be used to substantially enhance the efficiency of expression in cases where rod and/or cone -specific expression of a potentially therapeutic gene is desired. The *gfp*-containing rAAV stock was substantially free of both adenovirus and wild-type AAV, as judged by plaque assay and infectious center assay, respectively. Thus, highly purified, helper virus-free rAAV vectors can achieve high frequency tissue-specific transduction of terminally differentiated, postmitotic photoreceptor cells. These methods can be used as described herein for gene therapy to treat retinal diseases.

In a preferred embodiment the human rod opsin, or cone opsin, promoter analogous to the mouse sequence would be used. The appropriate regions of the human sequence can be readily identified and used by the skilled artisan having the benefit of the instant disclosure.

Assuming a 20 μl injection into the central human retina at the region of greatest rod density, approximately 8 million rods would be expected to be transduced in a focal region encompassing approximately 6% of the total retina. By transducing such an area of PRs it is possible to improve or delay retinal degenerations in a variety of inherited retinal diseases.

Currently, a number of PR genes of therapeutic potential have been identified experimentally by virtue of their involvement in recessive human retinal disease and by their ability at least to delay the course of recessive RP-like disease in animal models. These include PDE-B (Bennett, J., T. Tanabe, D. Sun, Y. Zeng, H. Kjeldbye, P. Gouras, A.M. Maguire (1996) *Nature Med.* 2:649-654; Lem, J., J. Flannery, T. Li, M. Applebury, D. Forber, M. Simon (1992) *Proc. Natl. Acad. Sci. USA* 89:4422-4426) and peripherin/rds (Travis, G.H., K.R. Groshan, M. Lloyd, D. Bok (1992) Neuron 33:113-119). Also included in this list are more general cell survival-promoting factors such as bcl-2 (Chen, J., J. Flannery, M. LaVail, R. Steinberg, J. Xu, M.I. Simon (1996) *Proc. Natl. Acad. Sci. USA* 93:7042-7047) and a variety of growth factors and neurotrophins (Faktorovitch, E., R.H. Steinberg, D. Yasumura, M. Matthes, M.M. LaVail (1990) 347:83-86). Thus, all forms of RP (Daiger, S., L. Sullivan, J. Rodriguez (1995) *Behav Brain Sci.* 18:452-467) described to date are potential candidates for therapy in this context.

In one specific embodiment, the subject invention utilizes the catalytic properties of ribozymes. Ribozymes are enzymes comprised of ribonucleic acid (RNA). In nature, ribozymes conduct a variety of reactions involving RNA, including cleavage and ligation of polynucleotide strands. The specificity of ribozymes is determined by base pairing (hydrogen bonding) between the targeting domain of the ribozyme and the substrate RNA. This specificity can be modified by altering the nucleotide sequence of the targeting domain. The catalytic domain of ribozymes, the part that actually performs the biochemical work, can also be changed in order to increase activity or stability of the ribozyme.

Utilizing the techniques of the subject invention, ribozymes are continuously produced in the retinal cells from a copy of the ribozyme integrated in the patient's DNA. In a preferred embodiment patients require a single intra-ocular injection and do not require hospitalization. Long term (more than 15 months) unattenuated expression of proteins has been observed in cells transformed as described herein.

In one embodiment the subject invention concerns synthetic genes for several ribozymes. These ribozymes can recognize, for example, the nucleotide change causing the P23H mutation in one form of ADRP and the S334ter mutation in another. Genes can be constructed which encode ribozymes having the ability to specifically destroy target RNA's for mutant retina proteins. With the benefit of the teachings provided herein, the skilled artisan can

construct genes encoding ribozymes which destroy mutant RNA molecules associated with human RP or other genetic retinal diseases.

Using a recombinant Adeno-associated virus (rAAV) in which expression is driven by a portion of the rod opsin promoter, we have achieved photoreceptor-specific expression of reporter genes in mouse and rat by ocular injection. The cone opsin promoter can also be used to drive expression selectively in photoreceptor cells. The delivery-expression materials and methods of the subject invention can be used to replace any gene responsible for photoreceptor disease. Specific examples include the genes responsible for retinitis pigmentosa or macular degeneration. General survival-promoting genes such as growth factor and neurotrophin genes are also candidates for both recessive and dominant forms of retinal disease. Finally, genes for agents such as ribozymes or triplex-forming oligonucleotides that can be designed to eliminate specific genetic defects are candidates for treating retinal disease using this technique. Assays for activity include morphological analysis of retinal degeneration, quantitative mRNA studies, and electroretinography.

Materials and Methods

rAAV plasmid construction. The mOp-*lac*Z -rAAV plasmid DNA was made by first inserting the 4.3 kbp Bgl II/Bam HI fragment containing the proximal murine rod opsin promoter (+86 to -385) and the entire *lac*Z gene of clone pRG3 (Lem, J., M. Applebury, J. Falk, J. Flannery, M. Simon (1991) *J. Biol. Chem.* 266:9667-9672) into the Bgl II sites of pTR which contains the AAV TR sequences and a SV40 polyadenylation sequence (Fig. 1a). The mOp-gfp-rAAV plasmid DNA was made by first adding Not I linkers to the 472bp Bgl II/Xho I proximal opsin promoter fragment of pRG3 and inserting it into the Not I sites of pTRUF2 (Zolotukhin, S. M. Potter, W. Hauswirth, J. Guy, N. Muzyczka (1996) *J. Virol.* 70:4646-4654) (Fig. 1b).

rAAV virus production and analysis. To generate recombinant virus, human 293 cells were co-transfected with mOp-*lac*Z-rAAV or mOp-*gfp*-rAAV plasmid DNA and the helper pIM45 plasmid DNA carrying the wtAAV genome without terminal repeats (Zolotukhin, S. M. Potter, W. Hauswirth, J. Guy, N. Muzyczka (1996) *J. Virol.* 70:4646-4654). Cultures were then infected with helper Adenovirus, Ad-ts149 for the *lac*Z virus or with Ad5 for the *gfp* virus, at a multiplicity of infection of 10 rAAV and wtAAV titers were determined by infectious center assay (McLaughlin, S. P. Collis, P. Hermonat, N. Muzyczka (1988) *J. Virol.* 62:1963-1973), which is independent of the transgene or opsin promoter used. Titers of contaminating adenovirus were determined by plaque assay for mOp-*gfp*-rAAV and by serial dilution

cytopathic effect for mOp-*lac*Z-rAAV. Adenovirus was not detectible in either of the rAAV preparations.

Subretinal injection of rAAV. Thirty adult C57BL/6I (Jackson Laboratories, Bar Harbor, ME) pigmented mice between 3 and 6 months of age and 27 adult albino Sprague-Dawley rats between 3 and 4 months of age were used. Animals were anesthetized by ketamine/xylazine injection, eyes were dilated (2.5% phenylephrine and 0.5% tropicamide) and a local anesthetic (proparacain Hcl) was applied. Injections (1 μl in mice and 2 μl in rats) were made into the right eye with blunt 32 gauge needle through an opening in the pars-plana, delivering the rAAV suspension into the superior subretinal space. Control injections were made in the contralateral eye with PBS only. Injections were performed with an operating microscope and the subretinal location of the injected volume was confirmed by ophthalmoscopy.

Tissue analysis. Animals were euthanized by intramuscular injection of ketamine, followed by phenobarbital overdose. The eyes were immediately enucleated and the site of virus injection marked. The cornea, lens and vitreous of each eye were removed and the posterior eyecup placed in primary fixative.

For β-galactosidase staining, eyecups were fixed in 0.5% glutaraldehyde in 0.1M Cacodylate buffer pH 7.5 for 15 min. At room temperature. Following a 10 min. Wash in PBS, the eyecups were incubated in an iron-based X-gel staining solution (Sanes, J., J. Rubenstein, J. Nicolas (1986) *EMBO J.* 5:3133-3142) in a shaking water bath at 35°C for 12 hours. For agarose embedment, retinas were detached from the RPE, submerged without dehydration in molten 5% agarose and cooled to 25°C. Retinas were sectioned in the transverse axis in isotonic PBS on a vibratome at 50-100 μm. Bright field and phase-contrast micrographs of whole mounts and β-galactosidase-stained sections were made with a Zeiss Axiophot.

GFP fluorescence was examined in retinal whole mounts and agarose embedded sections. Tissue fixation was minimized to reduce retinal autofluorescence. Retinas were detached from eyecups, fixed for 15 min. At room temperature in 4% formaldehyde, 0.1 M PO_4 buffer pH 7.5, and rinsed three times in PBS. Whole mounts were photographed with epifluorescence using Zeiss filter set 09 (ex. 450-490 nm, barrier 510 nm, emission 520 nm) and an AttoArc (Carl Zeiss, Inc., New York) variable output UV lamp to minimize GFP bleaching. Whole mount retinas were then embedded in agarose as above for 100 μm transverse vibratome sections, and fluorescence was documented as for the whole mount. Higher resolution images were collected with a Molecular Dynamics confocal microscope (Nikon 40X or 60X 1.4 n.a. oil objectives; argon laser excitation at 514 nm, emission at 520-560 nm). Optical sections were

made in 0.32 μm steps. Full frame (768 x 512) 8-bit images were collected and processed with Adobe Photoshop. Area measurements were made with NIH Image analysis software (Rasband, W. D. Bright (1995) *Microbeam Analysis Society Journal* 4:137-149).

Expression of the *lacZ* reporter gene in murine retinal cells was analyzed by reverse transcriptase PCR (RT-PCR). Pieces of retina (1mm^2), were detached from unfixed eyecups and dissected free of RPE, homogenized with a pestle fitted to a 1.5 ml tube and total RNA isolated using the trizol reagent (phenol-guanidine isothiocyanate, Gibco-BRL, Gaitherberg, MND) according to the manufacturer's recommendations. The RNA was additionally purified over an RNA-easy spin column (Qiagen, Chatsworth, CA). The RT-PCR employed a two buffer thermostable Tth polymerase system (Promega, Madison, WI) according to manufacturer's instructions and *lacZ* sequence primers from nucleotides 105 to 124 (forward) and 303 to 286 (reverse). Rnase and Dnase digestions prior to the RT-PCR were performed as previously described (van Ginkel, P., W. Hauswirth (1994) *J. Biol. Chem.* 269:4986-4992).

Following are examples which illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 – Design of rAAV Vectors for Gene Transfer to Photoreceptors

To express a foreign gene specifically in the mammalian PR by AAV-mediated delivery, a 472bp of the proximal murine rod opsin promoter (+86 to -385) was linked to a lacZ-SV40 polyA reporter gene and then inserted this into pTR. The gene construct was packaged into AAV virus particles, concentrated, tested for contaminating Adenovirus and titered for recombinant AAV by an infectious center assay. The right eyes of 30 C57Bl/6J mice were injected sub-retinally with 1μl of mOp-*lacZ* virus (10^7 iu per ml). After two weeks, the right (test) and left (control) eyes of 12 animals were removed, fixed and stained with X-gal. Test retina in 6 of 12 injected eyes exhibited a focal blue region consistent with a subretinal bleb of the injected virus creating a localized retinal detachment. All control eyes showed no X-gal reaction. Reporter gene expression was examined in mice sacrificed at later periods and was detected at 10 weeks post-injection suggesting persistent reporter transgene expression.

Example 2 – Lac-Z and GFP Reporter Genes are Expressed Exclusively in Photoreceptors

The distribution of lacZ gene product was analyzed at higher resolution by preparing serial 50 μm transverse sections from the entire whole mounts. The blue X-gal reaction product

is observed primarily in the PR inner segments. Most of the PRs were filled with X-gal in this region. X-gal staining was slightly above control levels in the PR synaptic termini in the outer plexiform layer. PR outer segments, RPE and other retinal cells in this region did not reveal X-gal staining above baseline levels observed in identically treated, uninjected or PBS-injected control retinus from the contralateral eye. Examination of additional transverse sections confirmed that the region of positive staining radiated outward from the injection site in a progressively reducing fraction of PR inner segments until baseline levels were seen. The area of X-gal positive PRs was consistent with the blue area in the whole-mount view. Neural retina and RPE were separated and analyzed independently to control for the possibility that the β-galactosidase enzyme or its X-gal reaction product was transferred from transduced RPE cells to PRs. Total mRNA was extracted from neural retina, and RPE from injected animals and tested for the presence of *lacZ* mRNA by RT-PCR. The 199bp amplification product diagnostic for *lacZ* RNA (nucleotides 105 to 303) can be seen when total RNA from a portion of a mouse retina sacrificed at 2 weeks post-injection is amplified. The amplification template was a cellular RNA because of its resistance to Dnase pretreatment and sensitivity to Rnase pretreatment. The remaining RPE tissue was negative for this RT-PCR product. This demonstrates that the observed X-gal product was derived from β- galactosidase expression within PR cells and not derived from RPE expression.

A second reporter gene, a synthetic version of the A victoria green fluorescent gene (gfp) (Zolotukhin, S. M. Potter, W. Hauswirth, J. Guy, N. Muzyczka (1996) *J. Virol.* 70:4646-4654) was used to independently confirm the apparent cell-type specificity of transduction. The same murine rod opsin promoter was used as well as an analogous rAAV vector to construct the mOp-gfp virus (Fig. 1b). Two μl of gfp-containing rAAV was injected into the subretinal space of 8 Sprague-Dawley rats. Rats were used in place of mice because the larger eye allowed more reproducible subretinal inoculations. Retinal whole mounts prepared from all eight rat eyes that were injected contained a fluorescent region of superior retina surrounding the site of inoculation. GFP fluorescence typically extended over 10-20% of the retinal area in a radial pattern from the injection site. Immediately surrounding the point of infection, the transduction frequency, as judged by the intensity of GFP fluorescence, was very high, with a continuous positive signal. In transverse sections extending from the central retina to the periphery, beyond a region of apparently saturated GFP fluorescence, the percentage of transduced cells decreased radially with distance from the injection site. GFP-positive cells were easily identifiable as PRs by their specialized shape and location in the retina. Hence, only PR cells appeared to have been transduced, i.e., infected by the rAAV and expressing the *gfp* passenger gene.

Example 3 – Opsin Promoter Confers Photoreceptor Cell Specificity

The PR-specific pattern of GFP expression was confirmed by laser confocal microscopy. GFP was not observed between the inner limiting membrane (vitreal face of the inner retina) and the outer plexiform layer (OPL) (junction of the inner retina with PR synaptic termini). This region contains all the non-PR retinal neuronal (bipolar, horizontal, amacrine, and ganglion) and glial (Müller) cells. Virtually 100% of the PR inner segments, cell bodies, and synaptic terminals exhibited strong GFP fluorescence. In regions more peripheral to the injection site, the fraction of positive PRs was substantially reduced, consistent with the radial decline in fluorescence seen in retinal whole mounts. It was established that all PR cell bodies contained GFP signal by examining serial optical sections (0.32 μm). Through-focus series demonstrated that occasional, dark regions in the ONL always contained a gfp-positive PR cell body in another plane of section. Therefore, all PRs, including both rods and cones, supported reporter gene expression. Outer segments demonstrated less fluorescence than other PR compartments, near the level of autofluorescence seen in control outer segments. No GFP signal was observed in the REP, choroid, or sclera.

Example 4 – rAAV Transduces PRs with High Efficiency

The area of GFP-positive PRs resulting from a typical injection from epifluorescence images of retinal whole mounts was established. GFP-positive areas were measured with NIH Image software by segmenting the image into regions of GFP fluorescence and background on the basis of gray level. Area measurements were calibrated by imaging a 1000 μm reference scale on the film together with the whole mount.

The retinal area that contained 50% or more PR cells positive for GFP signal in whole mounts was measured. On average, the GFP-positive area covered ~35% of the total retinal area of the rat retina. The number of GFP-positive PRs resulting from a typical injection was estimated by examining serial optical sections taken through the retina. Serial confocal images suggest 100% PR transduction in the region directly adjacent to the injection site, since we did not observe GFP-negative cell bodies within the outer nuclear layer in adjacent confocal optical sections. It is estimated that the whole rat retina contains 15.7 million PR cells. From these observations, a conservative estimate is that 2-3 million PRs were transduced by the *gfp*-containing rAAV. Since there were 9.2 million infectious rAAV particles in the 2-μl injection volume, one PR cell was transduced for every 3-4 rAAV injected, a 25% transduction efficiency.

Example 5 – Construction of Vectors and Expression in Target Cells

rAAV-ribozyme constructs. Recombinant AAV constructs were based on the pTR-UF2 vector (Zolotukhin, S., M. Potter, W.W. Hauswirth *et al.* [1996] *J. Virol.* 70:4646-4654). They resemble the vector used by Flannery *et al.* (Flannery, J.G., S. Zolotukhin, M.I. Vaquero *et al.* [1997] *Proc. Natl. Acad. Sci. USA* 94:6916-6921) to direct GFP expression to rat photoreceptors except that a 691 bp fragment of the proximal bovine rod opsin promoter replaced the 472 bp murine rod opsin promoter and the ribozyme gene replaced the *gfp* gene. The bovine promoter fragment contains three proximal promoter elements and the endogenous transcriptional start site at its 3′ end (DesJardin, L.E., W.W. Hauswirth [1996] *Inv. Ophth. Vis. Sci.* 37:154-165) and supports high efficiency, rat photoreceptor-specific expression *in vivo*. Active and inactive ribozymes were designed, tested and cloned. Each ribozyme gene was followed by an internally cleaving hairpin ribozyme derived from plasmid pHC (Altschuler, M., R. Tritz, A.A. Hampel [1992] *Gene* 122:85-90) resulting in ribozyme cassettes of 140-152 bp. Self cleavage at the internal cutting site in the primary ribozyme RNA leaves identical 3′ ends on each mature ribozyme. The ribozyme cassette was preceded by an intron derived from SV40 and followed by a polyadenylation signal in order to promote nuclear export of the ribozyme. Recombinant AAV titers were determined using both an infectious center assay (Flannery, J.G., Zolotukhin, S. Vaquero *et al.* [1997] *Proc. Natl. Acad. Sci. USA* 94:6916-6921) and a DNAse resistant physical particle assay employing a quantitative, competitive PCR of the *neo*^{r} gene contained within all rAAV-ribozyme particles (Zolotukhin, S., M. Potter, W.W. Hauswirth *et al.* [1996] *J. Virol.* 70:4646-4654). Each of the four rAAV-ribozyme virus preparations contained 10^{10} to 10^{11} DNASE resistant particles per ml and 10^8 to 10^9 infectious center units per ml. Contaminating helper adenovirus and wild-type AAV, assayed by serial dilution cytopathic effect or infectious center assay respectively, were less than five order of magnitude lower than rAAV.

Subretinal injection of rAAV. Line 3 albino transgenic rats (P23H-3) on an albino Sprague-Dawley background (produced by Chrysalis DNX Transgenic Sciences, Princeton, NJ) were injected at the ages of P14 or P15. Animals were anesthetized by ketamine/xylazine injection, and a direction, and b-waves were measured from the cornea-negative peak to the major cornea-positive peak. For quantitative comparison of differences between the two eyes of rats, the values from all the stimulus intensities were averaged for a given animal.

Retinal tissue analysis. The rats were euthanized by overdose of carbon dioxide inhalation and immediately perfused intracardially with a mixture of mixed aldehydes (2% formaldehyde and 2.5% glutaraldehyde). Eyes were removed and embedded in epoxy resin, and

1 μm thick histological sections were made along the vertical meridian (26). Tissue sections were aligned so that the ROS and Müller cell processes crossing the inner plexiform layer were continuous throughout the plane of section to assure that the sections were not oblique, and the thickness of the ONL and lengths of RIS and ROS were measured as described by Faktorovich *et al.* (Faktorovich, E.G., R.H. Steinberg, D. Yasamura *et al.* [1990] *Nature* 347:83-86). Briefly, 54 measurements of each layer or structure were made at set points around the entire retinal section. These data were either averaged to provide a single value for the retina, or plotted as a distribution of thickness or length across the retina. The greatest 3 contiguous values for ONL thickness in each retina were also compared to determine if any region of retina (*e.g.*, nearest the injection site) showed proportionally greater rescue; although most of these values were slightly greater than the overall mean of all 54 values, they were no different from control values than the overall mean. Thus, the overall mean was used in the data cited, since it was based on a much larger number of measurements.

RT-PCR. For quantification of opsin mRNA retina from ribozyme injected or control eyes, retina were isolated without fixation and total RNA immediately extracted using the RNeasy Minikit (Qiagen, Santa Clarita, CA). RT-PCR was performed using the Pharmacia First-Strand cDNA synthesis kit employing oligo dT as the primer. Wild-type and transgene opsin cDNAs were amplified using a three primer system described above. Primers specific for β-actin cDNA (Timmers, A.M., B.R. Newton, W.W. Hauswirth [1993] *Exp. Eye Res.* 56:251-265) were included in each reaction for internal standardization.

Such constructs result in persistent photoreceptor expression of the passenger gene of greater than 15 months. Ribozymes were designed to recognize and cleave the unique transcript produced by the P23H transgene. The mutant target sequence "5′-UCGGAGUCUACUUCG-3′" (SEQ ID NO. 17) contains two differences from the wild-type mRNA (indicated in bold). The hairpin ribozyme (Hp11) cleaved 3′ to the first adenosine residue (underlined) and the hammerhead ribozyme (Hh13) cleaved 3′ to the central cytosine residue (underlined). Control ribozymes (Hp11i and Hh13i, respectively) retained the targeting domains but contained fatal flaws in their catalytic domains. *In vitro*, the active hammerhead ribozyme (Hh13) was able to cleave 20% of the P23H target within 10 min. of incubation and by 5 hours greater than 80% was converted to the expected products. In multiturnover experiments, both ribozymes exhibited kinetic constants (K_m and k_{cat}) similar to those of naturally occurring ribozymes. The two active ribozymes produced negligible cleavage of the wild-type transcript even in the presence of high $MgCl_2$ concentrations. Control ribozymes (Hp11i and Hh13i) containing inactivating mutations in their catalytic domains were without measurable activity on any substrate. Using total RNA

derived from retinas of P23H rats on P62, both the hairpin and the hammerhead ribozymes were able to cleave the mRNA product of the mutant transgene selectively.

For experiments *in vivo*, a line of transgenic rats, TgN(P23H)3 (abbreviated P23H-3), that has a retinal degeneration phenotype similar to patients with retinitis pigmentosa (Steinberg, R.H., J.G. Flannery, M.I. Naash *et al.* [1996] *Inv. Ophth. Vis. Sci.* 37:S698) was used. Expression of the mutated opsin transgene begins at about postnatal day (P) 5 in rats, leading to a gradual death of photoreceptor cells. These rats develop an apparently normal retina up to P15, although there are somewhat more pyknotic photoreceptor nuclei in the outer nuclear layer (ONL) than in non-transgenic control rats. Thereafter, death of photoreceptor cells is almost linear until about P60, resulting in loss of about 40% of the photoreceptors. After P60, the rate of cell loss decreases, until by one year the retinas have less than a single row of photoreceptor nuclei. The rAAV-ribozyme vector was injected into the interphotoreceptor space between the photoreceptors and the adjacent retinal pigment epithelium at P14 or P15. Rats were sacrificed and eyes examined at 3 time points between P60-P90. At these ages in uninjected control eyes of P23H-3 rats, the ONL thickness, which is an index of photoreceptor cells number, was reduced to about 60% of normal.

Ribozyme-injected eyes showed a modest but significant decrease in the accumulation of transcript derived from the P23H transgene. Control eyes exhibited little variation in the level of transgene opsin mRNA. Eyes injected with either active ribozyme uniformly exhibited lowered transgene mRNA levels relative to total opsin mRNA in the same eye. Retinas receiving the hairpin ribozyme Hp11 showed a 15.3±3.3% decrease in transgene expression, and those with the hammerhead ribozyme Hh13 showed a decrease of 11.1±5.1% decrease.

Histologically, eyes injected with the ribozymes retained significantly more photoreceptors at P60, P75 and P90 than uninjected contralateral control eyes. Retinas receiving a subretinal injection of Hh13 at P14-15 retained 88% of the normal ONL thickness, compared to about 60% in the uninjected controls (Figure 2a). Thus, the ONL thickness after Hh13 expression was 40-43% greater than that of uninjected P23H-3 controls (Figure 2b), a highly significant difference ($p=0.001$ or less at P60 and P90). Injection of the Hp11 ribozyme also resulted in significant rescue when compared to controls, with preservation of 77-83% of normal ONL thickness (Figure 2a). Thus, the ONL thickness after Hp11 expression was 30-39% greater than that of uninjected P23H-3 controls (Figure 2b), a highly significant difference ($p<0.0005$ at all ages).

There was little or no rescue in PBS-injected control eyes ($p>0.169$ in all cases) as shown in Figure 2a. As a control for possible rescue by the expression of the bovine opsin

promoter (BOPS), AAV-BOPS-*gfp* was injected at a titer of 1.75×10^8, similar to the titer used with the AAV-ribozymes. The injection of AAV-BOPS-*gfp* did not rescue photoreceptors (Figure 2a). The inactive Hp11i did yield ONL thickness measures greater than uninjected control values, but they were consistently less than that resulting from the active Hp11 and Hh13 ribozymes (Figure 2a).

The pan-retinal extent of photoreceptor rescue that resulted from a single 2-μl injection of the rAAV suspension was surprising (Figure 2b). From photoreceptor counts, it is estimated that there are approximately 10^7 photoreceptors in the rat retina. Recombinant AAV titers were estimated using both an infectious center assay and a physical particle assay. Together they permit construction of upper and lower bounds for the number of functional rAAV particles in a single 2 μl injection. The upper bound derives from the DNAse resistant particle assay, indicating that 2 μl of the rAAV-ribozyme virus preparation contained 2×10^7 to 10^8 rAAV. This is an upper bound because not all particles counted are expected to be infectious. The lower bound for rAAV titer is generated by the infectious center assay, indicating 10^6 to 10^7 rAAV per μl.

The lateral extent of rescue resulting from a single injection may also be explained by the unique nature of the retinal tissue. For *in vivo* delivery to the photoreceptors, rAAV is injected into extracellular space separating the photoreceptor and retinal pigment epithelium (RPE) layers. The initial volume of extracellular space, approximately 0.5 μl, increases greatly with the 2 μl injection. Following injection, the fluid transport function of the RPE dehydrates this space, reapposing the photoreceptors and RPE and concentrating the rAAV. The detachment of the photoreceptors from the RPE resolves within several hours. During the reattachment process, viral particles are spread laterally through the subretinal space.

Along with the survival of more photoreceptor cells, injection of the ribozymes resulted in greater lengths of rod inner segments (RIS) and rod outer segments (ROS). In the case of RIS, the uninjected control retinas had RIS that were about 90% of normal. Both the active and inactive ribozymes resulted in RIS lengths of 98% or greater of the normal length, and about 10-15% longer than uninjected controls. The PBS and AAV-BOPS-*gfp* were indistinguishable from uninjected eyes. The ROS lengths were about 15-25% longer in the ribozyme-injected eyes compared to those in the uninjected control eyes. However, ROS in the ribozyme-injected eyes were, at greatest, only 65-75% of normal, compared to the virtually normal RIS lengths. The ROS of the active ribozymes differed significantly from the uninjected controls ($p<0.005$ for all, except <0.02 for Hp11 at P90), as did the inactive Hp11i ($p<0.05$).

The finding that ribozyme-targeted destruction of P23H mutant RNA markedly slows the rate of retinal degeneration in P23H transgenic rats, along with functional preservation of the retina, is the first demonstration of this therapeutic approach in an animal model of a dominantly inherited human disease. Furthermore, because complete removal of mutant rRNA is not necessary to achieve phenotypic rescue, this approach can be applied to other dominantly inherited diseases as well.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

Claims

1. A method for expressing a polynucleotide at high levels specifically in photoreceptor cells wherein said method comprises administering to said photoreceptor cells a construct comprising said polynucleotide wherein said polynucleotide is under the control of a promoter sequence which directs expression only in said photoreceptor cells.

2. The method, according to claim 1, wherein said cells are rod cells.

3. The method, according to claim 1, wherein said cells are cone cells.

4. The method, according to claim 1, wherein the promoter is a rod opsin promoter.

5. The method, according to claim 1, wherein the promoter is a cone opsin promoter.

6. The method, according to claim 1, wherein said polynucleotide sequence is delivered to said retina cells by subretinal injection.

7. The method, according to claim 1, wherein said polynucleotide sequence is delivered using a recombinant Adeno-Associated Virus (rAAV).

8. The method, according tro claim 1, which is used to treat retinal disease.

9. The method, according to claim 8, wherein said retinal disease is selected from the group consisting of Retinitis Pigmentosa and Macular Degeneration.

10. A construct for expressing a polynucleotide sequence selectively in photoreceptor cells wherein said construct comprises said polynucleotide sequence under the control of a promoter which directs expression selectively in photoreceptor cells.

11. The construct, according to claim 10, wherein said promoter is a rod opsin promoter.

12. The construct, according to claim 10, which comprises a recombinant Adeno-Associated Virus (rAAV).

13. The construct, according to claim 10, wherein said polynucleotide sequence is for the treatment of a retinal disease.

14. The construct, according to claim 13, wherein said retinal disease is selected from the group consisting of Retinitis Pigmentosa and Macular Degeneration.

15. The construct, according to claim 10, wherein said promoter is a rod opsin promoter.

16. The construct, according to claim 10, wherein said promoter is a cone opsin promoter.

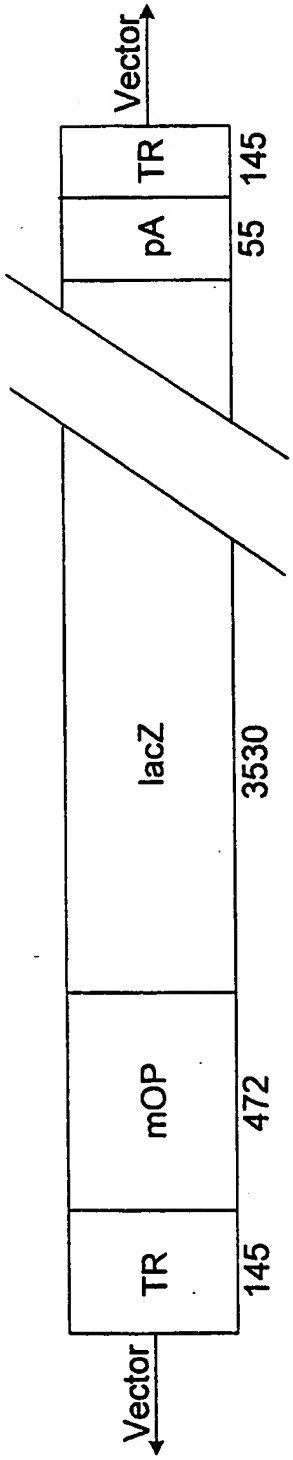
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FIG. 1A

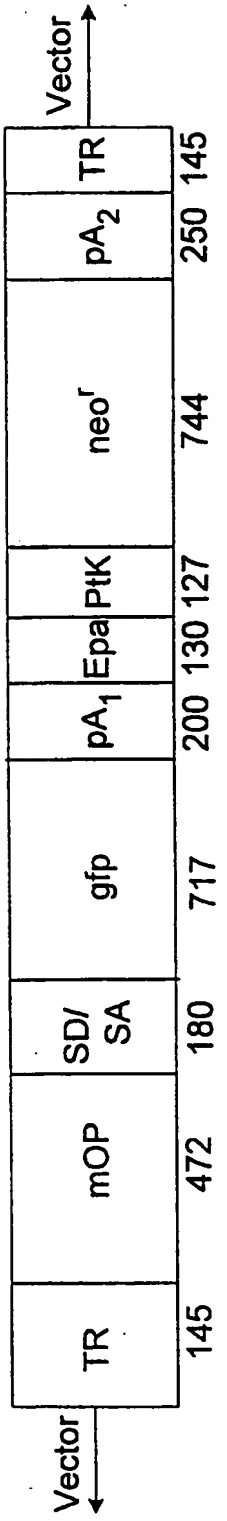
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FIG. 1B

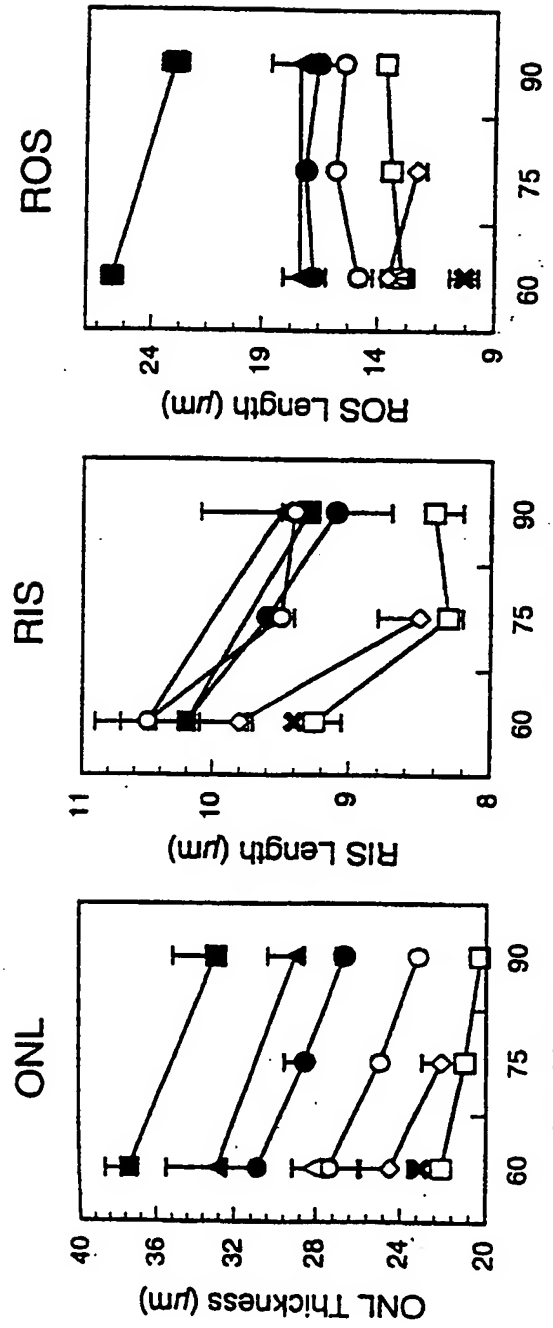

FIG. 2A

FIG. 2B

FIG. 2C

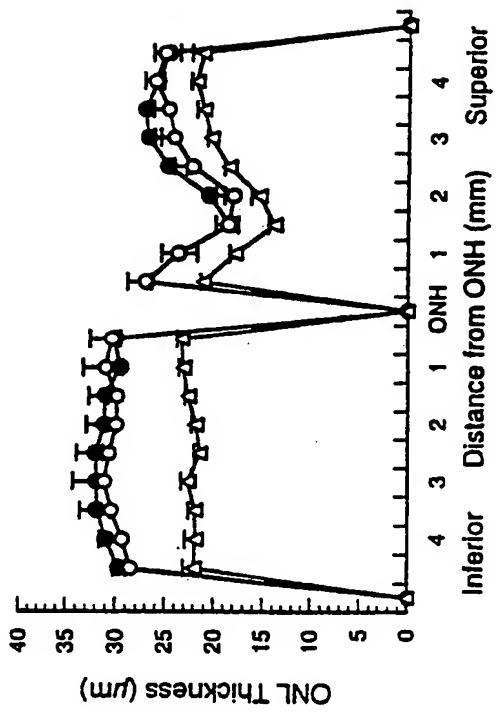

FIG. 2D